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(71) Applicant: MARTEK BIOSCIENCES CORPORATION [US/US]; 6480 Dobbin Road, Columbia, MD 21045 (US).

(72) Inventor: KYLE, David, John; 1801 Narberth Road, Catonsville, MD 21228 (US).

(74) Agents: ERNST, Barbara, G. et al.; Rothwell, Figg, Ernst & Kurz, Suite 701 East, 555 13th Street, N.W., Washington, DC 20004 (US).

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(54) Title: METHOD OF TREATING CORONARY VASCULAR DISEASE USING DOCOSAHEXAENOIC ACID

(57) Abstract

A method of treating coronary vascular disease in a human including the administration of a serum cholesterol reducing effective amount of DHA-containing single cell oil to the human.

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METHOD OF TREATING CORONARY VASCULAR DISEASE USING DOCOSAHEXAENOIC ACID

Field of the Invention

This invention relates to a method of treating coronary vascular disease in a human by administering a serum cholesterol reducing effective amount of docosahexaenoic acid (DHA) to said human. The DHA is administered in the form of a single cell oil containing DHA in the form of a triglyceride.

Background of the Invention

Hypercholesterolemia is defined by the presence of excess cholesterol in the blood. High levels of blood cholesterol can be a major risk factor in the premature development of atherosclerosis. It follows that hypercholesterolemia is a major risk factor for the development of coronary vascular disease. Accordingly, reduction of blood cholesterol levels in humans can be useful in preventing both atherosclerosis and coronary vascular disease.

A number of epidemiological studies have correlated diets enriched in fish oils with a low incidence of coronary vascular disease. Populations that have been studied have included the Greenland Eskimos, Danes, Canadian Inuit and Japanese. Further investigations subsequent to these early observations which focused on possible mechanisms responsible for this effect have found that the level of omega-3 long

chain polyunsaturated fatty acids (omega-3 PUFAs) in the diet is a significant determining risk factor.

Fish oils comprise a complex mixture of bioactive fatty acids, including two omega-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The relative amounts of each of these two omega-3 PUFAs can vary, depending upon the species of fish.

In several recent studies, researchers have administered the ethyl ester form of either DHA or EPA separately to laboratory animals to investigate the effects of each in the absence of the other. The administration of the ethyl ester form of DHA and EPA to rats has been reported by Ikeda et al., in Wasugi, T., Nakamura, H., and Soma, M., Advances in Polyunsaturated Fatty Acids Research, Excerpta Medica Amsterdam, pp. 223-226 (1993). Ikeda et al. reported that DHA in the ethyl ester form decreased plasma cholesterol levels and EPA in the ethyl ester form decreased plasma triglyceride levels in rats. Great Britain Patent Application No. 2,098,065 also has reported that the ethyl ester form of DHA, which was obtained by esterifying the free fatty acid isolated from fish oil, lowers blood cholesterol levels in rats.

Unfortunately, the results obtained from experiments like those described in Ikeda et al. and Great Britain Patent Application No. 2,098,065, in which DHA and EPA are administered to non-human mammals, are not predictive of the effect which DHA and EPA, either in ethyl ester form or other lipid form, will have in humans. For example, Harris et al., "Chylomicronemia is Reduced by Fish Oil but not by Pure Eicopentaenoic Acid in Hypertriglyceridemics," AAP/ASCI/APCR Abstracts in Joint Sessions, 401A, A59

report that the decrease in serum triglyceride levels which results from the administration of fish oil (containing DHA and EPA as triglycerides) to human patients does not appear to be similar to the effects of purified EPA free acid alone.

Moreover, the purified ethyl ester forms of EPA or DHA are very difficult to make in commercially relevant quantities. They also are very expensive, rendering them unsuitable for wide-spread commercial application.

There thus remains a need in the art for methods of treating coronary vascular disease in humans.

Summary of the Invention

The present invention relates to a method of treating hypercholesterolemia in a human by administering a serum cholesterol reducing effective amount of DHA to said human. The DHA is in the form of a DHA-containing single cell edible oil. As used herein, "single cell edible oil" refers to a lipid product of a unicellular organism. More specifically, it refers to a lipid product in triglyceride form.

Brief Description of the Drawings

Each of Figures 1 and 2 is a graphic illustration of *C. cohnii* biomass accumulation over time with the addition of various nutrients.

Detailed Description of the Preferred Embodiments

In accordance with the present invention, coronary vascular disease is treated by administering to a human patient in need of such treatment a serum cholesterol reducing effective amount of DHA so as to reduce the amount of cholesterol in the patient's blood. The DHA is in the form of a triglyceride and is obtained as a single cell oil by the cultivation of a DHA-producing microorganism under oil-producing conditions.

According to preferred embodiments of the present invention, microorganisms capable of producing a single cell oil containing DHA are cultivated in a fermentor in a nutrient solution capable of supporting the growth of such organisms. Preferably, the single cell oil is enriched in DHA, meaning it will contain at least about 20% by weight DHA.

Any microorganisms capable of producing a single-cell edible oil containing DHA can be used in the present invention. Microorganisms which can be used can be identified by the presence of DHA in the fatty acid profile of the biomass. For example, species of photosynthetic algae such as *Chattonella, Skeletonema, Thalassiosira, Isochrysis, Hymenomonas, Euglena* and *Cryptomonas* can be used. Preferred microorganisms are heterotrophic species of algae which include, but are not limited to the Dinophyceae (e.g., *Crypthecodinium*), or fungi such as Chytridiomycetes (e.g., *Thraustochitrium* and *Schitzochytrium*) or Oomycetes (e.g., *Mortierella, Saptrolegnia* and *Mucor*).

In accordance with the present invention, preferred microorganisms produce oils containing DHA and essentially no EPA (i.e., less than about 5% EPA). Producing oils with essentially no EPA to be administered to patients to treat coronary vascular

disease is desirable because (1) as EPA is known to be a blood thinning agent, the administration of a microbial oil containing EPA is undesirable to patients whose blood is already thin or where an increased prothrombin time may be contraindicated (e.g., elderly patients, hypertensive patients, pregnant women, etc.); and (2) there are some indications that EPA may actually increase blood cholesterol levels in humans. See, e.g., Bonaa, K. et al., "Habitual Fish Consumption Plasma Phospholipid Fatty Acids, and Serum Lipids: the Tronso Study," Am J. Nutr., 55(6), pp. 1126-34 (1992). An especially preferred microorganism is *Crypthecodinium cohnii* which, as described in U.S. patent application serial number 07/479,135, filed February 13, 1990, is an obligate heterotroph requiring a reduced carbon source for growth. *C. cohnii* is especially preferred because it contains a natural oil with a fatty acid profile in which DHA is abundant and the only long chain (i.e,. at least 20 carbon atoms) polyunsaturated fatty acid present in significant quantities (greater than about 1% of the total amount of PUFAs). Thus, the DHA-containing oil produced by *C. cohnii* is essentially free of EPA and other PUFAs, which provides an advantage for using the microbial oil rather than fish oil to treat hypercholesteremia. Samples of one strain of *C. cohnii* which produces abundant levels of DHA, have been deposited with the American Type Culture Collection at Rockville, Maryland, in accordance with the provisions of the Budapest Treaty, and assigned accession number 40750.

As used herein, the term "microorganism", or any specific type of microorganism, includes wild strains, mutants or recombinant types. Any microorganism which produces enhanced levels of oil containing DHA is

considered to be within the scope of this invention. Accordingly, wild-type and recombinant microorganisms designed to produce single cell oil containing DHA are an aspect of this invention. Such recombinant organisms would include those designed to produce greater quantities of DHA in the single cell oil, greater quantities of total oil, or both, as compared to the quantities produced by the same wild type microorganism, when provided with the same substrates. Also included would be microorganisms designed to efficiently use more cost-effective substrates while producing the same amount of single cell oil containing DHA as the comparable wild-type microorganism.

The DHA-producing microorganisms can be cultivated in a simple medium containing a carbon source, such as glucose, and a nitrogen source, such as yeast extract. Use of these components in a suitable nutrient solution such as seawater provides economically significant growth rates and cell densities. For example, during the course of a 3-5 day fermentation, microorganism cell densities of at least 10 grams of biomass per liter of solution, and typically from 20 to about 40 grams per liter, can be attained.

Although cultivation can occur in any suitable fermentor, preferably the organism is grown either in a stirred tank fermentor (STF) or in an air lift fermentor (ALF), both types known to those of skill in the art. When a STF is selected, agitation is provided using either Rushton-type high efficiency turbines or pitched-blade or marine impellers. Agitation and sparging renew the supply of oxygen to the microorganisms. The rate of agitation normally is increased as the biomass increases, due to the increased demand for oxygen. It is desirable to keep

the tip speed at not greater than about 500 cm/sec, preferably not greater than about 300 cm/sec. Selection of strains of microorganisms which are capable of withstanding greater tip speeds without undergoing shear is within the purview of those of skill in the art. The use of such strains is expressly included in this invention.

As noted above, seawater is an acceptable medium for the nutrient solution. The seawater can be either natural, filtered or an artificial mix, each of which can be diluted to reduced salinities, such as 1/2 to 1/4 normal strength, with tap water or concentrated to 2 times normal strength. *C. cohnii* can be grown, for example, in Instant Ocean® (IO) brand artificial seawater, or a mixture of NaCl (4.5-20 g/L), $MgSO_4 \cdot 7H_2O$ (1.23 g/L), and $CaCl_2 \cdot 2H_2O$ (90 mg/L) in water.

Some growth of marine microorganisms has been observed in very low salinity. Micronutrients known to those of skill in the art can be added and may be required at such low salinities. If the organism selected is heterotrophic, such as *C. cohnii*, then a carbon source is added.

Preferably, after addition of the medium containing the nutrient solution to the fermentor, the fermentor containing the medium is sterilized and cooled prior to adding the nutrients and a seeding population of microorganism. (Although it is acceptable to sterilize the nutrients together with the nutrient solution, sterilization in this manner can result in a loss of available glucose.) The nutrients and microorganism can be added simultaneously or sequentially.

An effective seed concentration can be determined by those of skill in the art. When a STF is used, the

addition of a population of from about 0.05 to 1.0 grams of dry weight equivalent per liter at the beginning of the fermentation is preferred. This is about 1-5 x 10^6 cells per ml. Thus, for a 30 liter fermentor, 1-3 liters of seeding media, containing viable cells at a density of 10-20g dry weight per liter would be added.

Oxygen levels preferably are maintained at a D.O. of at least about 10% of air saturation level. Biosynthesis of DHA requires oxygen and, accordingly, higher yields of DHA require D.O. levels at from about 10% to 50% of air saturation levels. Agitation tip speeds of 150-200 cm/sec in combination with an aeration rate of 1 VVM (volume of air/volume of fermentor per minute) provides D.O. levels of from about 20% to about 30% at biomass densities of about 25 g dry weight/liter of culture. Higher cell densities may require higher D.O. levels, which can be attained by increased aeration rates by O_2 sparging, or by increasing the back pressure in the fermentor.

Acceptable carbon sources are known to those of skill in the art. For example, carbon can be provided to microorganisms in the form of mono- or di-saccharides, such as glucose and sucrose. Other heterotrophs can use other reduced carbon sources, a matter easily determined by those of skill in the art, and autotrophs utilize carbon dioxide. Microorganisms will also grow on other reduced, more complex, carbon sources. Typically, a fermentation is initiated with about 10-50 g/liter glucose. More glucose is added during the fermentation as required. Alternatively, from about 50 to 150 g, preferably 50 to 100g glucose/liter initially can be added, thereby minimizing the frequency of future additions. The

amount of carbon source provided to other organisms can readily be determined by those of skill in the art.

In addition to a reduced carbon source, a nitrogen source, such as yeast extract (YE) or peptone, is provided to the medium. Commercially available yeast extract or peptone is acceptable. For example, Difco or Marcor brand yeast extract or Sheftone brand peptone can be used. Yeast extract and peptone are organic nitrogen sources which also contain micronutrients. Other organic nitrogen sources easily can be determined by those of skill in the art. However, such compounds are generally more expensive than yeast extract. Variants capable of growing on urea, ammonia or nitrates also can be used.

Typically, the fermentation is initiated with about 6-12 g YE/liter. More YE can be added as required. A typical fermentation run requires from about 8 to 15 g YE/liter over the course of the run. Accordingly, that amount of YE can be added initially with a reduced need for further additions. The precise amount can be determined by those of skill in the art. Generally, the ratio of glucose to YE is from about 2:1 to about 25:1.

The cultivation can be carried out at any life-sustaining temperature. Some algae and fungi have an effective temperature range of from about 10-40°C. Generally, *C. cohnii*, for example, will grow at temperatures ranging from about 15°C to 34°C. Preferably, the temperature is maintained at about 20-30°C. Strains which grow at higher temperatures are preferred, because they will have a faster doubling time, thereby reducing the fermentation time. Appropriate temperature ranges for other microorganisms are readily determined by those of skill in the art.

The cultivation can be carried out over a broad pH range, typically from about pH 5.0 to 9.0. Preferably, a pH range of from about 6.0 to about 7.0 is used for the growth phase. A base, such as KOH or NaOH, is used to adjust the media pH prior to inoculation. During the later stages of the fermentation, the culture medium pH may increase or decrease. If desired, acid or base pH controls can be used to correct alkalinity during the growth phase.

Production of the single cell oil is induced in the microorganisms by the imposition of a stationary phase (e.g., by nitrogen depletion, phosphate depletion or a pH rise). Nitrogen deficiencies are caused by providing the nitrogen source, such as YE, in a limiting amount such that the medium runs out of YE while available glucose remains. It is the carbon source to nitrogen source ratio which promotes the efficient production of the single cell oil. Using glucose and YE as exemplary, a preferred ratio of carbon source to nitrogen source at inoculation is about 10-15 parts glucose to 1 part nitrogen source. Similar ratios for other carbon and nitrogen sources can be calculated by those of skill in the art.

After induction of oil production, the culture is grown for about 24 additional hours. During this period of oleosynthesis, the single cell oil containing DHA is being synthesized and visible oil droplets become apparent within the cells. Those of skill in the art can readily calculate the time of fermentation required to achieve the expected amount of cell biomass based upon the added amount of nitrogen source. When that time has passed, the culture is grown for an additional 24 hours and harvested. In general, the microorganisms are cultivated for a time sufficient to

produce single cell oil, usually from about 60 to about 90 hours, although this time is subject to variation.

From about 15 to 30% of the resultant biomass comprises extractable oil. Strain selection can increase this percentage and such selection is within the capabilities of one with ordinary skill in the art. Preferably, the oil comprises greater than about 70% triglycerides. For *C. cohnii*, for example, the oil can have, in general, the following fatty acid composition.

15-20% myristic acid ($C_{14:0}$)
20-25% palmitic acid ($C_{16:0}$)
10-15% oleic acid ($C_{18:1}$)
30-50% DHA ($C_{22:6}$)
0-10% others

(Other oil components including polar lipids, such as phosphatidyl choline, also can be enriched in DHA.) The crude oil is characterized by a yellow-orange color and is liquid at room temperature. Desirably, the oil contains at least about 20% DHA by weight, preferably at least about 40% DHA by weight, and most preferably about 50% DHA by weight.

The organisms are harvested by conventional means, known to those of skill in the art, such as centrifugation, flocculation or filtration, and can be processed immediately or dried for future processing. In either event, the oil can be extracted readily with an effective amount of solvent. Suitable solvents can be determined by those of skill in the art. However, preferred solvents include pure hexane and supercritical fluids, such as supercritical CO_2. Certain lipophilic antioxidants (such as carotene, tocopherol, ascorbyl palmitate, BHT, etc.) can be added prior to extraction. These compounds help protect the

oil from oxidation during the extraction and refining processes.

General extraction techniques using supercritical fluids have been developed for oil extraction from oil-rich plant seeds (McHugh et al., Supercritical Fluid Extraction, Butterworth, 1986). However, these standard methods cannot be applied to the extraction of microalgae such as *C. cohnii* because spray dried algae cells have a consistency of flour with a relatively high bulk density and flow of supercritical CO_2 is restricted as this material is compressed. In addition, the cell walls of microalgae are chemically dissimilar to those of most seed oil material. In order to prevent the compression and allow efficient flow and extraction, the algal biomass can be mixed with from 0.1 to 5 parts of a lipid free structure agent, such as Celite, or diatomaceous earth. In a 50 ml reaction vessel at 450 Atm. and 100°C. 86% of the oil was extracted from *C. cohnii* in 25 minutes, and 100% was extracted in 85 min.

If the extraction solvent is hexane, a suitable ratio of hexane to dry biomass is about 4 liters of hexane per kilogram of dry biomass. The hexane preferably is mixed with the biomass in a stirred reaction vessel at a temperature of about 20-50°C for about 2 hours. After mixing, the biomass is filtered and separated from the hexane containing the oil. Alternatively, a wet biomass paste (30-35% solids) can be extracted directly with more polar solvents, such as ethanol, isopropanol or hexane/isopropanol mixtures.

The solvent then is removed from the oil by distillation techniques known to those of skill in the art. Conventional oilseed processing equipment is suitable to perform the filtering, separation and

distillation. Additional processing steps, known to those of skill in the art, can be performed if required or desirable for a particular application. These steps also will be similar to those involved in conventional vegetable oil processing and allow the separation of DHA-enriched polar lipid fractions.

According to preferred embodiments of the present invention, compositions containing DHA can be formulated in a conventional manner for administration by any suitable route. Suitable routes of administration include, but are not limited to, oral, nasal, topical, and parenteral (including subcutaneous, intramuscular, intravenous and intradermal), with oral or parenteral being preferred. It will be appreciated that the preferred route can vary with the condition and age of the recipient.

While not essential, it is preferable for the DHA oil to be administered as part of a pharmaceutical formulation. The formulations of the present invention comprise DHA together with one or more pharmaceutically acceptable carriers and optionally with other therapeutic ingredients. The carrier(s) are "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The formulations include those suitable for oral, nasal, topical, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations conveniently can be presented in unit dosage form, e.g., emulsions, tablets and sustained release capsules, and can be prepared by any suitable pharmaceutical methods.

Such methods include, but are not limited to, the step of bringing DHA oil into association with the

pharmaceutically-acceptable carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association DHA with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations of the present invention suitable for oral administration can be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of DHA; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion, etc. The oils also can be administered by adding the purified and sterilized oil to a prepared enteral formula which then is placed in the feeding tube of a patient who is unable to swallow. Preferably, formulations of the present invention are administered via gel capsules or oil-in-water liquid emulsions. Example 4 below describes one way to produce suitable gel capsules for administration of the formulations of the present invention. However, it will be recognized that any known means of producing gel capsules can be used in accordance with the present invention.

A tablet can be made by compression or molding the DHA oil with one or more pharmaceutically-acceptable, dry, inert accessory ingredients such as carboxymethylcellulose. Compressed tablets can be prepared by compressing in a suitable machine DHA, mixed with a powder or granules, binder, lubricant, inert diluent, preservative and/or surface-active or dispersing agent in accordance with conventional tableting procedures. The tablets optionally can be coated or scored and can be formulated so as to provide

slow or controlled release of the active ingredient therein.

Other formulations suitable for oral administration include lozenges comprising DHA in a flavored basis, such as sucrose and acacia or tragacanth; and pastilles comprising DHA in an inert basis such as gelatin and glycerin, or sucrose and acacia.

Suitable formulations for oral administration of DHA containing oils also include dietary supplements and/or food products made with DHA containing oils. For example, DHA containing oils can be incorporated into food products such as cookies or biscuits, and these food products can be given to patients in need of coronary vascular disease treatment. Furthermore, DHA containing oils can be used to supplement, or can be used instead of, typical cooking oils during the preparation of food products and/or dietary supplements.

Formulations suitable for topical administration to the skin can be presented as ointments, creams, gels and pastes comprising DHA and a pharmaceutically acceptable carrier. A preferred topical delivery system is a transdermal patch containing the oil to be administered.

In formulations suitable for nasal administration, the carrier is a liquid, such as those used in a conventional nasal spray or nasal drops.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which optionally can contain pharmaceutically-acceptable anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient and

aqueous and non-aqueous sterile suspensions which can include suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention can include other suitable agents having regard to the type of formulation in question. For example, those suitable formulations of DHA-containing oils for oral administration can include flavoring agents.

The compositions of the present invention are formulated into doses which will provide a serum cholesterol reducing effective amount of DHA. Typically, a useful daily dose is within a range from about 0.50 to about 100 mg per kg of body weight of recipient per day (mg/kg/day), preferably from about 5 to about 50 mg/kg/day.

The acceptable daily dose of DHA may be conveniently administered by giving the DHA oil in 1 to 3 doses per day. The precise dose administered will depend on the age and condition of the recipient.

Once a patient's blood cholesterol level has been brought within the normal range, as defined by the American Medical Association for the patient's sex and age, through the administration of the DHA-containing single cell oil, the patient desirably continues to

take the medication at a dose rate to maintain his cholesterol within a desirable range.

The invention having been generally described, the following examples are given as particular embodiments of the invention and to demonstrate the practice and advantages thereof. It is understood that these examples are given by way of illustration and are not intended to limit the specification or the claims to follow in any manner.

Example 1

Into a 350-liter working volume STF was loaded a medium of one half strength artificial seawater made by combining 4.3 kg. of I.O.® with 230 liters of tap water. The fermenter containing the medium was sterilized and cooled to 28°C. 6.8 liters of concentrated YE (400g/l), 12.5 liters of glucose syrup (400g/l) and 30 liters of *C. cohnii* inoculum from a seed fermenter (10^6 cells/ml or a biomass density of about 1.3g/liter) were added to the medium. Agitation was set at 73 cm/sec tip speed and aeration was set at 1 VVM (280 liters per minute). Additional glucose syrup (12 liters) was added after about 44 hours and another 43 liters over the next 32 hours. Thus, 67.5 liters of glucose syrup were added in total. The glucose additions and the cell growth are depicted graphically in Figure 1.

To maintain the D.O. at greater than 20%, at 44 hours the agitation tip speed was increased to 175 cm/sec and at 55 hours to 225 cm/sec. At 76 hours, the tip speed was decreased to 150 cm/sec and the culture was permitted to grow for an additional time sufficient to convert the final charge of glucose into cellular oil. The culture then was harvested. The harvested

cells were dried to about a 4% moisture content. Hexane was added to the dried biomass and stirred in a glass kettle for 2 hours at 25°C. A rotary evaporator was used to remove the hexane, producing about 700 g of crude DHA-containing oil.

Example 2

Into a 15,000 liter fermenter (10,000 L net volume) was loaded 60 kg of yeast extract, 45 kg of NaCl, 12.3 kg of $MgSO_4 \cdot 7H_2O$, and 0.9 kg of $CaCl_2 \cdot H_2O$ in 7,000 L of water. This solution was sterilized before adding 3,000 L of a separately sterilized glucose solution (650 kg glucose per 3,000 L). The initial pH was set to 6.3, the temperature to 28°C, aeration to 0.5-1.0 vvm, vessel back pressure to 0.2 bar, and agitation to 120 cm/sec (tip speed) before inoculating the vessel with 300 L of an inoculum culture of *C. cohnii* which had attained a cell density of about 6 x 10^6 cells/ml (4-5 g dry weight/L) in an inoculum tank. During the course of the fermentation, a good grade antifoam (e.g. Dow 1520) was added on demand and the pH was controlled at 6.3 using 8 N H_2SO_4 and 4 N NaOH. The dissolved oxygen level was maintained at greater than 20% of air saturation by increasing the vessel back pressure and agitation. Additional glucose feeds were required at 93 hr and 111 hr to maintain the glucose levels above 5 g/L. The course of fermentation is shown in Figure 2. At 119 hours, the fermentor was cooled to 17°C and the fermentation broth was processed through a centrifuge producing 608 kg of a slurry containing 25% solids. The slurry was spray dried producing about 150 kg dry algal powder which contained about 30-40 kg of oil with a DHA content of 40-45%.

The dry algal powder (5% moisture content) was extracted with hexane using standard vegetable oil extraction equipment and methods and desolventized crude oil was degummed by the addition of water at 50°C. The degummed oil was collected by centrifugation and refined by mixing with caustic and phosphoric acid at 55°C for one hr. The refined and degummed oil was then collected by centrifugation and gently bleached at 90°C by the additional of citric acid and bleaching clay. Filtration of the bleaching clay produced the refined/bleached oil (RB-oil) with a peroxide value of less than 5 mEq/kg. The RB oil then was deodorized by high vacuum short path distillation and the resulting deodorized RB-oil (RBD-oil) was then ready for encapsulation, tableting, or bulk shipping. The resulting oil had a peroxide value less than 1 mEq/kg, a free fatty acid content of less than 0.5%, a DHA content of 45-47%, and an iodine number of about 200.

Example 3

Into a 1.7 L STF was loaded 2.5 g NaCl, 5 g $MgSO_4 \cdot 7H_2O$, 1 g KCl, 0.1 g KH_2PO_4, 0.2 g $CaCO_3$, 0.2 g $(NH_4)_2SO_4$, 2 g sodium glutamate in 1 liter of water. After sterilization, a sterile solution containing 10 μg thyamine-Hcl, 0.1 g $NaHCO_3$, and 10μg vitamin b_{12} was added, followed by 150 ml of sterile 30% glucose and 50 ml of sterile 10% yeast extract. The pH was adjusted to 6.0, the sparging to 1.0 vvm, and agitation to 300 rpm before inoculation with 100 ml of a 5-day old shake flask culture of *Thraustochytrium areum* grown in the same medium. The culture was harvested after 9 days to yield about 4 g dry weight of biomass. The DHA content of the lipid in the biomass was 10-15%.

Example 4

The DHA-rich oil was prepared for oral use by either encapsulating or tableting. Clear sealed gelatin capsules of 1 g/capsule were prepared by conventional manufacturing methods. Banded gel caps containing DHA-rich microbial oil (45% DHA) were prepared by allotting 250 μL of oil in the gel cap bottoms using a positive displacement manifold pipetter. With this method weight accuracy of ±5% was attained. The tops were then placed over the gel caps and they were banded with dyed gelatin using a capsule banding machine. Alternatively, the gel cap bottoms were filled with carboxymethylcellulose (CMC) and 100 mg (120 μL) of oil were pipetted directly onto this binder where it was adsorbed preventing any leakage. The tops were then placed over the gel caps and they were banded with dyed gelatin using a capsule banding machine. Alternatively, the CMC was mixed with the oil (three parts CMC to one part oil) in a separate container and pressed into tablets using a tablet press.

Example 5

Treatment of hypercholesterolemia using DHA-rich oil from *C. cohnii* is initiated by providing 1000 mg gel caps (containing about 450 mg DHA per capsule) at a dose of 3-9 capsules per day, three times per day with meals. Treatment continues until serum cholesterol drops to normal levels for age and sex as determined by the American Heart Association. Once reaching normal cholesterol levels, a dose regimen of a single 1000 mg gel cap per day can be used in order to maintain the normal cholesterol levels.

What is claimed is:

1. A method of treating coronary vascular disease in a human which comprises administering to a human in need of such treatment a single cell oil comprising DHA in an amount effective to reduce the human's level of serum cholesterol.

2. The method of claim 1, wherein the oil comprises at least 20% DHA.

3. The method of claim 2, wherein the oil comprises at least 40% DHA.

4. The method of claim 1 or 2, wherein the oil is essentially free of EPA.

5. The method of claim 1, wherein said single cell oil is obtained by cultivating at least one heterotrophic species of algae under DHA-oil-producing conditions and recovering the oil produced.

6. The method of claim 5, wherein said heterotrophic species of algae is a dinoflagellate.

7. The method of claim 6, wherein said dinoflagellate is *C. cohnii*.

8. The method of claim 1, wherein said single cell oil is obtained by cultivating at least one fungus under DHA-oil-producing conditions and recovering the oil produced.

9. The method of claim 8, wherein said fungus is a Chytridomycetes.

10. The method of claim 9, wherein said Chytridomycetes is *Thraustochytrium* or *Schitzochytrium*.

11. The method of claim 1, wherein said single cell oil is obtained by cultivating at least one Oomycetes under DHA-producing conditions and recovering the oil produced.

12. The method of claim 5, wherein said Oomycetes is selected from the group consisting of *Mortierella*, *Saprolegnia*, and *Mucor*.

13. The method of claim 1, wherein the single cell oil is administered so as to provide DHA at a dosage from about 0.50 to about 100 mg per kg of body weight per day.

14. The method of claim 13, wherein the single cell oil is administered so as to provide DHA at a dosage from about 5 to about 50 mg per kg of body weight per day.

15. The method of claim 1, wherein the single cell oil is administered orally.

16. The method of claim 15, wherein the single cell oil is administered to said human in a capsule, a tablet or an emulsion.

17. The method of claim 1, wherein the single cell oil is administered parenterally.

18. The method of claim 1, wherein the single cell oil is administered topically.

19. The method of claim 1, wherein single cell oil is administered in a food product or dietary supplement.

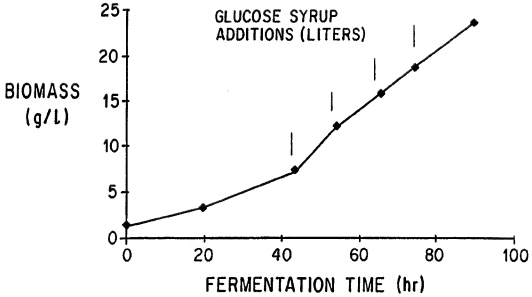
GLUCOSE SYRUP
ADDITIONS (LITERS)
BIOMASS
(g/L)
25
20
15
10
5
0
0
20
40
60
80
100
FERMENTATION TIME (hr)

FIG. 1

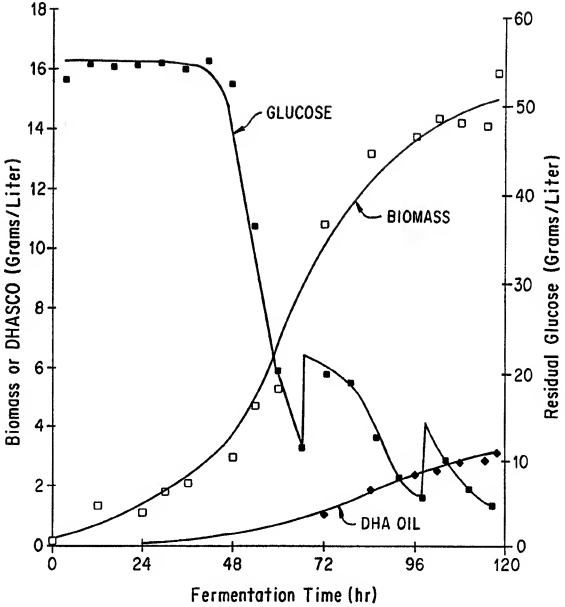
GLUCOSE
BIOMASS
DHA OIL
Biomass or DHASCO (Grams/Liter)
Residual Glucose (Grams/Liter)
Fermentation Time (hr)
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
50
60
0
24
48
72
96
120

FIG. 2

INTERNATIONAL SEARCH REPORT

Int. ional application No.
PCT/US94/06316

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 31/20
US CL :514/560,824
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/560,824

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS AND CAS ONLINE: DOCOSAHEXAENOIC ACID, CHOLESTEROL, CORONARY VASCULAR DISEASE, HEART DISEASE, ATHEROSCLERO?, DHA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,012,761 (OH) 07 May 1991, column 1 lines 9-47.	1-19
Y	US, A, 5,130,242 (BARCLAY) 14 July 1992, column 1, lines 23-28; column 4, line 52 - column 5, line 20; column 6, lines 6-54; column 10, lines 40-60; column 14, lines 59-68.	1-19
Y	GB, A, 2,098,065 (FUJITA ET AL.) 17 November 1982, page 2, lines 12-22 and 79-117.	1-19
Y	EXCERPTA MEDICA AMSTERDAM, issued 1993, Ikeda et al., "Differential Effect of Detary Docosahesaenoic and Eicosapentaenoic Acids on Lipid Metabolism In Rats", pages 223-26, abstract only.	1-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 AUGUST 1994	SEP 02 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KIMBERLY JORDAN Telephone No. (703) 308-1235

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